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Inhibition of Testosterone Production by Propylthiouracil in Rat Leydig Cells[1]

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ABSTRACT

Propylthiouracil (PTU) is a thioamide drug used clinically to inhibit thyroid hormone production. However, PTU is associated with some side effects in different organs. In the present study, the acute and direct effects of PTU on testosterone production in rat Leydig cells were investigated. Leydig cells were isolated from rat testes, and an investigation was performed on the effects of PTU on basal and evoked-testosterone release, the functions of steroidogenic enzymes, including protein expression of cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$) and mRNA expression of the steroidogenic acute regulatory protein (StAR). Rat Leydig cells were challenged with hCG, forskolin, and 8-bromo-cAMP to stimulate testosterone release. PTU inhibited both basal and evoked-testosterone release. To study the effects of PTU on steroidogenesis, steroidogenic precursor-stimulated testosterone release was examined. PTU inhibited pregnenolone production (i.e., it diminished the function of $P450_{scc}$ in Leydig cells). In addition to inhibiting hormone secretion, PTU also regulated steroidogenesis by diminishing mRNA expression of StAR. These results suggest that PTU acts directly on rat Leydig cells to diminish testosterone production by inhibiting $P450_{scc}$ function and StAR expression.

cyclic adenosine monophosphate, Leydig cells, signal transduction, testosterone, toxicology

INTRODUCTION

Propylthiouracil (PTU) is commonly used to treat hyperthyroidism conditions such as Graves disease. PTU inhibits thyroid hormone synthesis and the conversion of thyroxine to triiodothyronine. PTU is theoretically preferred over methimazole (MMZ, another thioamide drug) in Graves disease relapse during the postpartum period because of its lower milk:serum concentration ratio [1]. However, PTU is associated with various side effects, including transient leukopenia [2], jaundice, hepatomegaly [3, 4], and vasculitis [5, 6].

Although the effects of PTU treatment on male infertility are unclear, the alternations of male reproductive functions, including testosterone production caused by the administration of PTU, have been described. It has been shown that transient neonatal hypothyroidism induced by treatment with PTU increases testicular size, Sertoli cell number, and daily sperm production in adult rats and mice [7–9]. Although peripheral levels of testosterone in adult rats are unaffected by neonatal PTU-induced hypothyroidism [9–11], Hardy et al. [12] demonstrated that both basal and LH-stimulated testosterone production are reduced in Leydig cells from PTU-treated rats, parallel with a decline in the number of hCG-binding sites in Leydig cells. Furthermore, Leydig cells from PTU-treated rats produce less testosterone than controls following incubation with hydroxycholesterol [12]. These reports suggest that the increase in Leydig cell numbers after neonatal PTU treatment is counterbalanced by a permanent decrease in Leydig cell steroidogenic function, resulting in no net change in peripheral testosterone levels [12]. However, all of these alterations in male reproduction, including testosterone production in Leydig cells, were attributed to hypothyroidism caused by PTU. Whether PTU has a direct action on Leydig cell steroidogenic function is unclear.

We previously showed that PTU has a direct effect on steroidogenesis in rats. PTU decreased both the adrenocortical response to ACTH in vivo and corticosterone production by rat zona fasciculata-reticularis cells [13]. PTU also decreases the production of testosterone in rat testicular interstitial cells [14]. These thyroid hormone-independent results indicate that PTU acts directly on the steroidogenic organs.

In the steroidogenic tissues, steroidogenic cells are also regulated by cytokines released from ambient cells. In order to investigate the direct effects of PTU on steroidogenesis, it is necessary to isolate steroidogenic cells from other cells. In some investigations, a testis tumor cell line is used to avoid regulation by other cells (e.g., macrophages). However, there may be a cross-reaction between oncogene expression and steroidogenesis. Therefore, in the present study, we isolated Leydig cells from rat testes to examine the direct effects of PTU on the production of testosterone and to investigate the involved mechanisms, including the function and expression of the cytochrome P450 side-chain cleavage ($P450_{scc}$) enzyme and the steroidogenic acute regulatory (StAR) protein. Our hypothesis is that PTU may act directly on Leydig cells to inhibit steroidogenesis by regulating the function of $P450_{scc}$ and StAR.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 300–350 g were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial illumination daily (0600–2000 h) and food and water ad libitum. The investigations were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (National Academy of Sciences).

Materials

Bovine serum albumin (BSA), Hepes, Hanks balanced salt solution, Medium 199, sodium bicarbonate, penicillin-G, streptomycin sulfate, heparin, collagenase, PTU, MMZ, hCG, forskolin, 8-Br-cAMP, and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile), an inhibitor of

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3β-hydroxysteroid dehydrogenase (3β-HSD), was provided by Sanofi-Synthelabo, Inc. (Malvern, PA). Anti-$P450_{scc}$ antibody was provided by Dr. Bon-Chu Chung (Academia Sinica, Taipei, Taiwan, ROC), and anti-StAR antibody was provided by Dr. D.M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX). [^{3}H]Testosterone and [^{3}H]pregnenolone were obtained from Amersham Life Science Limited (Buckinghamshire, U.K.). [α-^{32}P]Deoxy-ATP was obtained from NEN Life Science Products (Boston, MA). The dosages of drugs are expressed in their final molar concentrations in the flask. The dosages of PTU used in this study were the same as those we used in a previous study [14]. Dosages were higher than the clinical dose (200–600 mg per person per day) [15, 16] but in the range of that used previously in rodent studies [17].

Preparation of Rat Leydig Cells

Animals were killed and the testes were collected and decapsulated. Testicular interstitial cells were isolated with the collagenase dispersion method as previously described [14]. The procedure used for preparing Leydig cells has been described elsewhere [18]. Testicular interstitial cells were centrifuged at 200 × *g* for 10 min at 4°C. The cell pellet volume was suspended in the incubation medium (1% BSA in Medium 199, with 25 mM Hepes, 2.2 g/ml sodium bicarbonate, 100 IU/ml penicillin-G, 50 μg/ml streptomycin sulfate, 2550 USP K unit/L heparin, pH 7.4, and aerated with 95% O_2 and 5% CO_2) to 5 ml and then added gently to the upper layer of the continuous Percoll gradient. The continuous Percoll gradient (20 ml/dispersion) was made by adding 9 parts of Percoll to 11 parts of 1.8× concentrated incubation medium before centrifugation at 20 000 × *g* for 60 min at 4°C. The mixture of testicular interstitial cells was loaded onto the Percoll gradient and centrifuged at 800 × *g* for 20 min at 4°C. Leydig cells were located in the 3- to 7-ml layer from the bottom. The Leydig cell layer was collected, diluted to 10 ml in incubation medium, and then centrifuged at 200 × *g* for 10 min at room temperature. After repeating the washing steps, the cell pellet was suspended to 10 ml in incubation medium. The cell concentration (2×10^5 cells/ml) and viability (over 95%) were determined using a hemacytometer and the trypan blue method. To measure the abundance of Leydig cells in our preparation, the 3β-HSD staining method was used [19, 20]. The cells (2×10^5 cells/ml) were incubated with a solution containing 0.2 mg/ml of nitro blue tetrazolium (Sigma) in 0.05 M PBS pH 7.4 at 34°C for 90 min. When the blue formazan deposit sites of 3β-HSD activity were developed, the abundance of Leydig cells was determined using a hemacytometer. Macrophages were determined by flow cytometry with fluorescein isothiocynate-conjugated monoclonal antibody (ED1, IgG 1; Biosource International, Foster City, CA). Our preparation contained approximately 87% Leydig cells and very few macrophages.

Effects of PTU on Testosterone Release by Rat Leydig Cells

Cells at a concentration of 2×10^5 cells/ml were preincubated at 34°C for 1 h in a controlled atmosphere (95% O_2 and 5% CO_2) and shaken at 100 cycles/min. The supernatant fluid was decanted after centrifuging the tubes at 100 × *g* for 10 min. The cells were then incubated with PTU (0, 3, 6, or 12 mM), MMZ (0, 3, 6, or 12 mM), or hCG (0.05 IU/ml) in 200 μl of fresh medium. Following 1 h of incubation, 1 ml of ice-cold 0.1% gelatin-phosphate-buffered saline pH 7.5 was added to stop the incubation. The medium was centrifuged at 100 × *g* for 10 min, and the supernatant was stored at −20°C until analyzed for testosterone by radioimmunoassay.

Aliquots (1 ml) of the cell suspensions (2×10^5 cells/ml) were challenged with PTU (0, 3, 6, or 12 mM) in the presence of hCG (0.05 IU/ml), forskolin (an adenylyl cyclase activator, 10^{-5} M), or 8-Br-cAMP (a membrane-permeable analogue of cAMP, 10^{-4} M) in 200 μl of fresh medium. At the end of incubation, the media were collected for testosterone radioimmunoassay.

Effects of PTU on the Functions of Steroidogenic Enzymes in Rat Leydig Cells

In this study, Leydig cells were incubated for 1 h with or without 12 mM PTU in the presence of five steroidogenic precursors (10^{-7} or 10^{-5} M). These precursors included 25-hydroxy-cholesterol (25-OH-C), a membrane-permeable cholesterol, pregnenolone (Δ_5P), progesterone, 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). At the end of incubation, the media were collected for testosterone radioimmunoassay.

Effects of PTU on Pregnenolone Production Stimulated with 25-OH-C in Rat Leydig Cells

Pregnenolone is the product of $P450_{scc}$ by conversion of cholesterol. Intracellular pregnenolone is metabolized by 3β-HSD. To investigate the production of pregnenolone we used trilostane (an inhibitor of 3β-HSD) to inhibit the catabolism of pregnenolone. Leydig cells (2×10^5 cells/ml) were preincubated with incubation medium for 1 h at 34°C. Cells were primed with trilostane (10^{-5} M) for 30 min and then incubated for 1 h with trilostane (10^{-5} M) or trilostane plus 12 mM PTU in the presence of 25-OH-C (10^{-7} or 10^{-5} M). At the end of incubation, the media were collected for pregnenolone radioimmunoassay.

For kinetic analysis of $P450_{scc}$, Leydig cells (2×10^5 cells/ml) were primed with trilostane (10^{-5} M) for 30 min and then incubated for 1 h with trilostane (10^{-5} M) or trilostane plus 6 mM PTU in the presence of 25-OH-C (10^{-6} to 10^{-4} M). At the end of incubation, the media were collected for pregnenolone radioimmunoassay.

Hormone Radioimmunoassays

Testosterone concentrations in media were determined by radioimmunoassay as described previously [21, 22]. The sensitivity of the testosterone radioimmunoassay was 2 pg per assay tube. The intraassay and interassay coefficients of variation were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentrations of pregnenolone were determined by radioimmunoassay as described previously [14]. The sensitivity of the pregnenolone radioimmunoassay was 16 pg per assay tube. The intraassay and interassay coefficients of variation were 2.3% (n = 6) and 3.7% (n = 4), respectively.

Western Blot Analysis

The Western blotting method has been described elsewhere [23, 24]. Leydig cells (1×10^6 cells/ml) were incubated with or without 6 mM PTU for 2 h. At the end of incubation cells were washed twice with ice-cold saline, then homogenized, and the protein was extracted in 50 μl of homogenization buffer (1.5% Na-lauroyl-sacrosine, 2.5 mM Tris base, 1 mM EDTA, and 0.1% PMSF, pH 7.8). The cell extract was centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatant was separated, and protein concentration was determined by modifying the Bradford protein assay method [25]. SDS-PAGE sample buffer (0.06 M Tris base, 2% SDS, 0.0005% bromophenol blue, 6% sucrose, and 50 μM dithiothreitol) was added to the samples, and after boiling for 5 min, the samples were stored at −20°C until they were used.

Samples (20 μg) were electrophoresed on a 12% minigel by standard SDS-PAGE procedures, along with prestained molecular weight markers (Bio-Rad, Hercules, CA). Gels were electrophoresed at 75 V for 15 min and then at 150 V for 30 min. The protein bands were transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA) with a semidry transfer cell (Bio-Rad) for 45 min as previously described [24]. The membrane was washed in TBS-T buffer (0.8% NaCl, 0.02 M Tris base, and 0.3% Tween 20 pH 7.6) for 5 min and blocked by a 120-min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). The membrane was then incubated overnight with anti-StAR (1:1000), anti-$P450_{scc}$ (1:2000), and anti-β-actin (1:8000). After three washes for 5 min each with TBS-T buffer, the membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (1:6000). Specific signals were detected by enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Life Science Limited).

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Leydig cells (5×10^6 cells/ml) were incubated with or without 6 mM PTU for 15, 30, and 60 min. Treatment with 8-Br-cAMP (10^{-4} M) was processed as a positive control. At the end of incubation, the cells were washed twice and total RNA was isolated with an RNAlarge (Blossom, Taipei, Taiwan, ROC) extraction kit (1 ml/5×10^6 cells). The procedures were conducted according to the manufacturer's instructions. RNA samples were dissolved in water containing 0.1% diethyl pyrocarbonate and quantified by measuring the absorbency at 260 nm. Aliquots containing 100 ng of RNA were assayed by the relative-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) procedure, which was modified from the method described by Ronen-Fuhrmann et al. [26]. Reverse transcriptase was conducted for 120 min at 37°C using 250 ng of pd(T) primer and 50 units of Moloney murine leukemia virus RT (BioLabs,

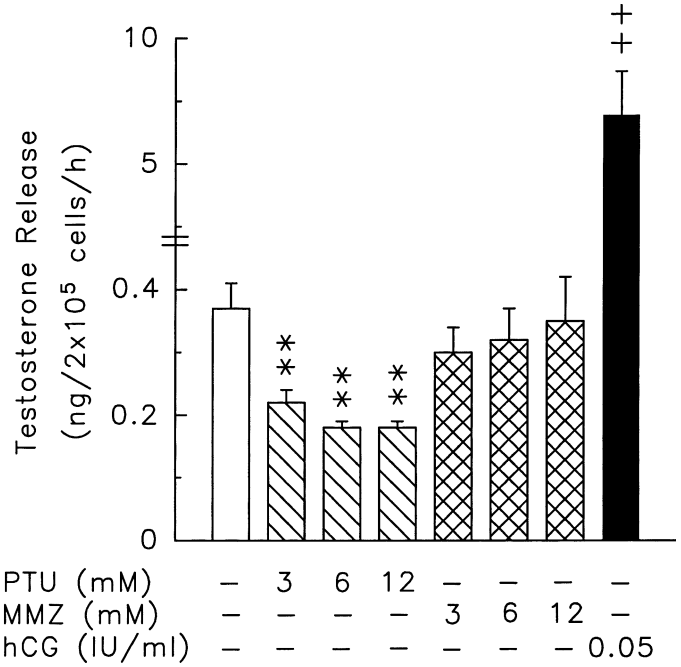

FIG. 1. Effects of PTU, MMZ, and hCG on basal testosterone release in rat Leydig cells. ++, $^{**}P < 0.01$ compared with the vehicle group. ++Significant increase. **Significant decrease.

Beverly, MA). The minus control of RT was processed to confirm that the mRNA samples were not contaminated by cellular DNA. PCR was performed in the presence of 2 μCi of [α-^{32}P]deoxy-ATP (3000 Ci/mmol), dNTPs (a mixture of dATP, dCTP, dGTP, and dTTP, 200 μM), and 500 ng of appropriate oligonucleotide primers. Oligonucleotide primers for the ribosomal protein L19 served as an internal control [26]. The number of cycles was examined to verify that the amplification was in the exponential phase. Following the PCR reaction (20 cycles), tracking dye was added to 10–40 μl of the PCR reaction mixture (100 μl) for analysis by 5% PAGE [27]. The gels were dried and exposed to x-ray film for 10 h and scanned with a scanner (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA). Quantification of scanned images was performed according to the ImageQuant program (Molecular Dynamics). The radioactivity in each PCR band was normalized to the radioactivity of the L19 band.

The PCR oligonucleotide primer pairs were designed based on known cDNA sequences of the various target genes. The expected PCR products would be 246 base pairs (bp) for rat StAR cDNA [26], 536 bp for rat $P450_{scc}$, and 194 bp for rat RPL19 [27]. Forward (A, sense) and reverse (B, antisense) primers were as follows: rat $P450_{scc}$ A, 5′-AGAAGCTGGGCAACATGGAGTCAG-3′, rat $P450_{scc}$ B, 5′-TCACATCCCAGGCAGCTGCATGGT-3′, rat StAR A, 5′-GCAGCAGGCAACCTGGTG-3′, rat StAR B, 5′-TGATTGTCTTCGGCAGCC-3′, RPL19 A, 5′-CTGAAGGTCAAAGGGAATGTG-3′, RPL19 B, 5′-GGACAGAGTCTTGATGATCTC-3′.

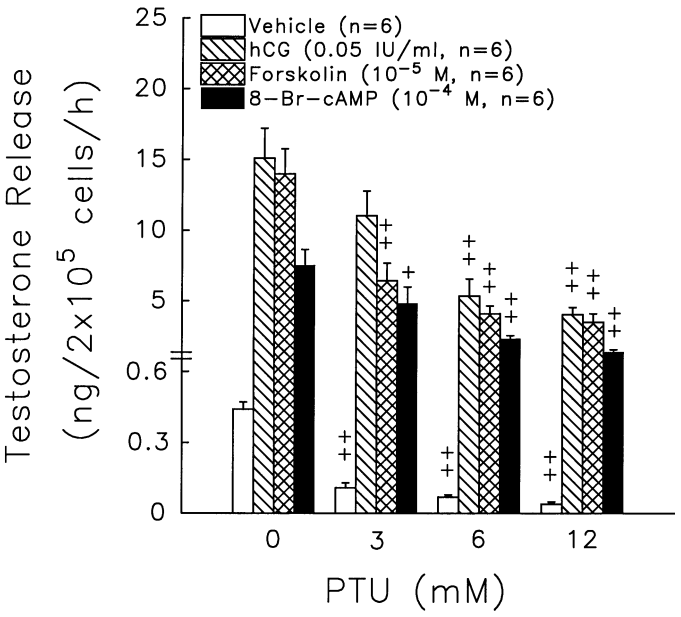

FIG. 2. Effects of PTU (3–12 mM) on basal and hCG-, forskolin- and 8-Br-cAMP-stimulated testosterone release in rat Leydig cells. Forskolin, hCG, and 8-Br-cAMP all significantly stimulated testosterone release ($P < 0.01$). $+P < 0.05$ and $++P < 0.01$ compared with PTU at 0 M.

Statistical Analysis

All values are given as the mean ± SEM. The treatment means were tested for homogeneity by one-way ANOVA and the differences between specific means were tested for significance by the Duncan multiple range test [28]. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Effects of PTU on Testosterone Secretion

Figure 1 shows that hCG significantly increased testosterone release in Leydig cells, and acted as a positive control for this study ($P < 0.01$). Administration of PTU (3–12 mM) inhibited basal testosterone release in Leydig cells ($P < 0.01$). However, another thioamide antithyroid drug, MMZ, did not alter testosterone production at the same doses.

In Figure 2, hCG, forskolin, and 8-Br-cAMP all stimulated testosterone release to a significant degree ($P < 0.01$). PTU (3–12 mM) inhibited not only basal but also hCG-, forskolin- and 8-Br-cAMP-stimulated ($P < 0.05$ or 0.01) testosterone release in Leydig cells.

Effects of PTU on Steroidogenesis in Leydig Cells

To investigate the effects of PTU on steroidogenesis, several steroidogenic precursors were used to challenge the Leydig cells (Fig. 3). The precursors included 25-OH-C (a substrate of $P450_{scc}$), pregnenolone (Δ_5P, a substrate of 3β-HSD), progesterone (P_4, a substrate of 17α-hydroxylase), 17α-hydroxy-progesterone (17α-OH-P, a substrate of C17-20 lyase), and androstenedione (Δ_4, a substrate of 17β-HSD). Two doses (10^{-7} and 10^{-5} M) of each precursor were employed. All precursors increased testosterone release in vitro. PTU (12 mM) decreased not only the basal release of testosterone but also the production of testosterone caused by the low (10^{-7} M) concentration of each precursor ($P < 0.01$ or 0.05). Meanwhile, PTU decreased the production of testosterone caused by the high concentration (10^{-5} M) of 25-OH-C ($P < 0.01$). However, PTU did not affect testosterone release induced by the same concentration (10^{-5} M) of the other 4 precursors tested.

Effects of PTU on Pregnenolone Production

In Figure 4, PTU (12 mM) decreased pregnenolone production evoked by 25-OH-C at 10^{-7} M and 10^{-5} M ($P<0.05$). A low concentration (10^{-7} M) of 25-OH-C evoked pregnenolone accumulation was diminished by PTU to an undetectable level.

In Figure 5, PTU (6 mM) decreased 25-OH-C evoked pregnenolone production from Leydig cells. The maximum velocities (V_{max}) were almost the same in the control and PTU groups (2.91 ng per h per 2×10^5 cells). The Michaelis constant (K_m) was 2.96 μM in the control group and 6.45 μM in the PTU group.

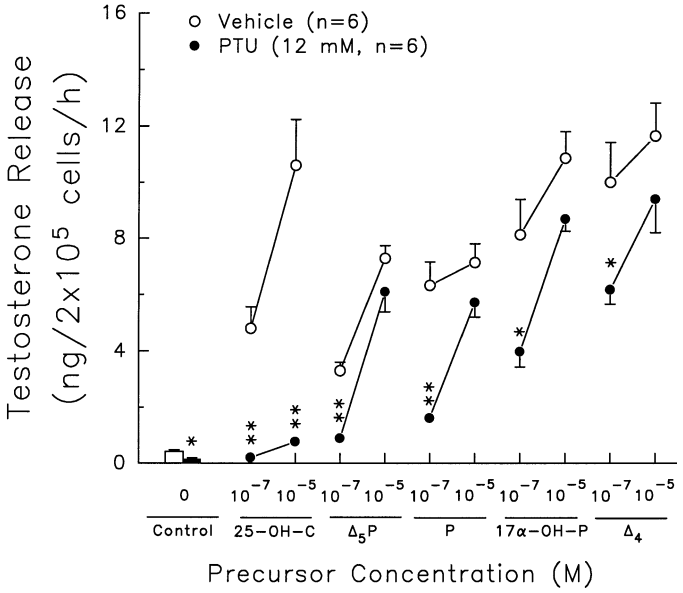

FIG. 3. Effects of PTU (12 mM) on steroidogenic precursor-stimulated testosterone release. The precursors include 25-OH-C, pregnenolone (Δ_5P), progesterone (P), 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). All the precursors significantly stimulated testosterone release ($P < 0.01$). *$P < 0.05$ and **$P < 0.01$ compared with the vehicle group.

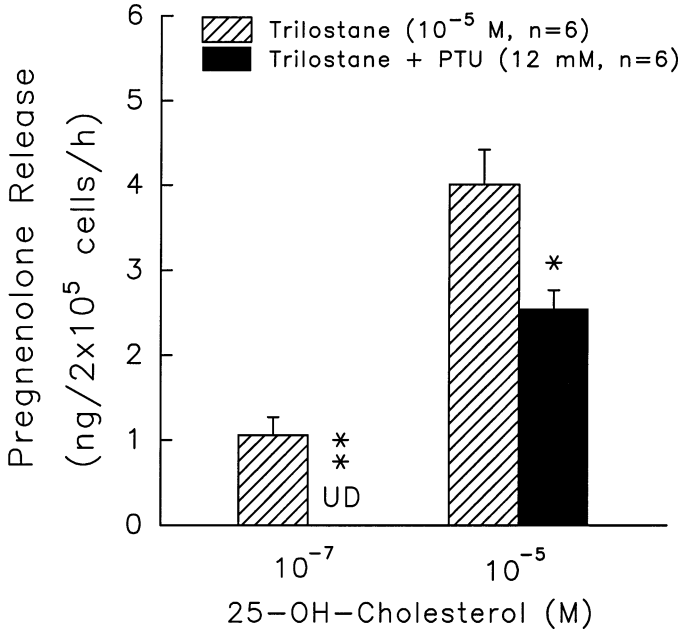

FIG. 4. Effects of PTU (12 mM) on 25-OH-C-stimulated pregnenolone release by Leydig cells in the presence of a 3β-HSD (a converter of pregnenolone to progesterone) blocker, trilostane (10^{-5} M). *$P < 0.05$ and **$P < 0.01$ compared with PTU at 0 M. UD, Undetectable.

Protein Expression of StAR and P450$_{scc}$

Bands at 54 kDa (P450$_{scc}$) and 30 kDa (StAR) were detected in rat Leydig cells (Fig. 6). The β-actin signal (45 kDa) was used as an internal control. Several independent experiments were repeated with similar results; however, there was no significant difference between the control and PTU groups.

Messenger RNA Expression of StAR and P450$_{scc}$

The objective of this experiment was to investigate StAR and P450$_{scc}$ expression in rat Leydig cells challenged with PTU. In our results, L19 was not affected by in vitro treatment. It is interesting that after the Leydig cells were challenged with PTU (6 mM), the level of StAR mRNA dropped markedly to 72% in 15 min and to 59% in 30 min (Fig. 7). This decrease in StAR mRNA level returned to normal after 1 h (data not shown). There was no significant change in the P450$_{scc}$ mRNA level within the 1-h period (data not shown).

DISCUSSION

PTU is widely used to treat patients with hyperthyroidism and in animals to induce hypothyroidism. Many results of metabolic dysfunction caused by the administration of PTU are attributed to hypothyroidism rather than the direct effect of PTU. We found, by accident, that some physiological responses might be induced by PTU, rather than by hypothyroidism. Our previous studies have shown that rat testicular interstitial cells exposed to PTU at pharmacological dosage levels in vitro diminishes basal and evoked testosterone secretion [14]. This indicates that the effects of PTU on testis are clearly independent of any effects of the drug on the secretion of gonadotropin or thyroid hormones. However, the composition of testicular interstitial cells and Percoll-purified Leydig cells are quite different. The interstitial cell preparation contains only approximately 20% Leydig cells. Testicular macrophages are another major population in the testicular interstitium (approximately 20%–30%) in addition to Leydig cells [29], and in our preparation of testicular interstitial cells (approximately 65%). Macrophages are acknowledged to be a major source of cytokines that may regulate other cells in the testis. Several studies indicate that macrophages play an important role in regulating steroidogenesis in Leydig cells [30, 31]. This study showed an inhibitory effect of PTU on Percoll-purified Leydig cells. Because our Leydig cell preparation was found to contain approximately 87% Leydig cells and very

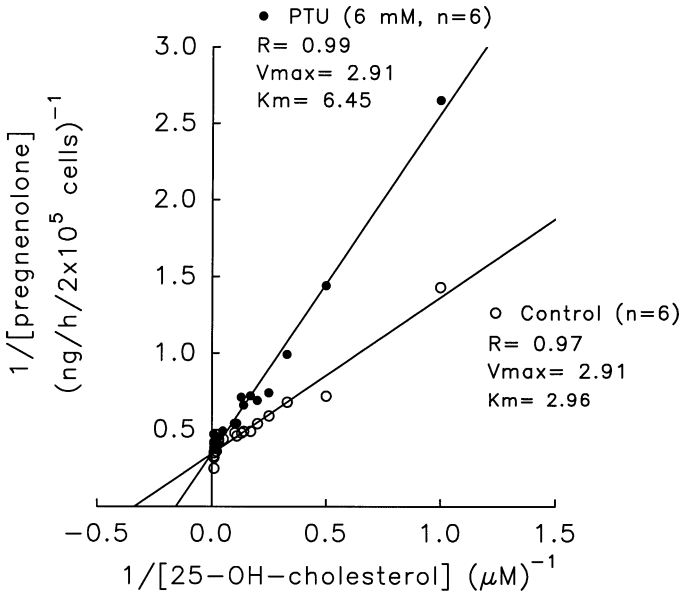

FIG. 5. Kinetic analysis of PTU inhibition of P450$_{scc}$ function. Double reciprocal plots of data were obtained from cultured Leydig cells challenged with 25-OH-C (10^{-6} to 10^{-4} M). V_{max} for both groups was nearly the same (2.91 ng per h per 2×10^5 cells). K_m for the PTU-treated group (6.45 μM) was greater than for the control group (2.96 μM).

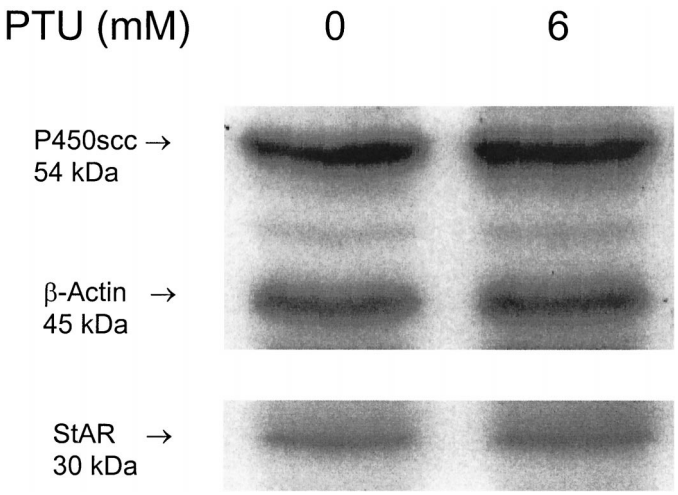

FIG. 6. $P450_{scc}$ and StAR protein expression under PTU treatment. Western blot analysis of cell extracts (20 μg) subjected to SDS-PAGE and developed by enhanced chemiluminescence. $P450_{scc}$ (54 kDa) and StAR (30 kDa) were detected by a concomitant incubation with $P450_{scc}$ and StAR antisera. Levels of $P450_{scc}$ and StAR protein were not affected by PTU (6 mM) treatment. This experiment was repeated three times with similar results.

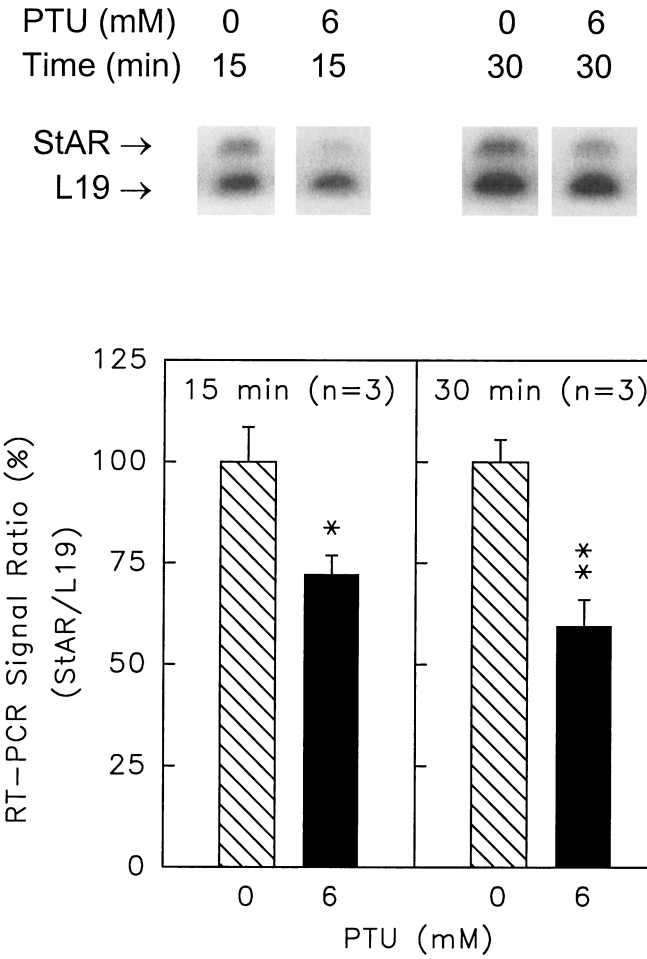

FIG. 7. PTU (6 mM) diminished StAR mRNA expression in Leydig cells. After rat Leydig cells were challenged with PTU for 15 min and 30 min, RNA was extracted, and 100 ng of RNA were assayed by relative-quantitative RT-PCR. Ribosomal protein L19 served as an internal control. Control groups were not treated with PTU. Top panels, autoradiograms depicting the amplified PCR signals obtained for each set of primers. PhosphorImager results are shown in the bottom panels. $*P < 0.05$ and $**P < 0.01$ compared with PTU at 0 M. The autoradiogram data are representative of three independent experiments repeated with similar results.

few macrophages, PTU would seem to act directly on the Leydig cells, and its effects are macrophage-independent.

Both PTU and MMZ are thioamide drugs used clinically to inhibit thyroid function. The clinical dose of PTU is approximately 10-fold greater than that of MMZ. However, MMZ did not regulate testosterone secretion at the same dose that PTU did (Fig. 1), which indicates that not all thioamide drugs have inhibitory effects on testis; rather, they may be PTU-specific.

PTU has often been used in long-term therapy, however, its acute side effects in pharmacological doses are not well known. To investigate the mechanism of PTU on testosterone secretion, we used an established in vitro model based on the ability of hCG to stimulate testosterone secretion [32, 33] via a mechanism involving cAMP production [32, 34, 35]. Our data showed that PTU inhibits not only basal but also hCG-, forskolin- and 8-Br-cAMP-stimulated testosterone release (Fig. 2). These results suggest that PTU acts directly on Leydig cells to regulate testosterone production at a point distal to the formation of cAMP.

In Leydig cells, testosterone is synthesized by several metabolic steps, collectively known as steroidogenesis. First, cholesterol is transformed to pregnenolone by $P450_{scc}$, the rate limiting enzyme. In rats, pregnenolone is then dehydrogenated to progesterone by 3β-HSD, progesterone is hydroxylated to 17α-hydroxy-progesterone by 17α-hydroxylase, and then 17α-hydroxy-progesterone is converted to androstenedione by C17-20 lyase (i.e., the Δ^4 pathway). Finally, androstenedione is reduced to testosterone by 17β-HSD. In our study, Leydig cells were incubated in vitro with the precursor of each metabolic step. Cholesterol was replaced by 25-OH-C because the latter is membrane-permeable. A higher concentration (10^{-5} M) was used to obviate the interference of any endogenous precursor. Because we observed the end product, testosterone, the functions of steroidogenic enzymes must be examined first from the last enzyme, 17β-HSD. The inhibitory effect of PTU was detected at a lower concentration (10^{-7} M) of androstenedione but not at a higher concentration (10^{-5} M). This indicates that the function of 17β-HSD may not be affected by PTU. A similar effect was found for the challenges with 17α-hydroxy-progesterone and progesterone. This supports the idea that the function of $P450_{c17}$ (which is involved in both the 17α-hydroxylase and C17-20 lyase reaction) is not affected by PTU (Fig. 3).

When Leydig cells were challenged with pregnenolone, PTU inhibited testosterone production at 10^{-7} M but not at 10^{-5} M. These data suggest that the function of 3β-HSD was also not affected by PTU. However, PTU inhibited 25-OH-C-stimulated testosterone production at both low and high concentrations, suggesting that the function of $P450_{scc}$ may be reduced by PTU.

In order to confirm that $P450_{scc}$ function is altered by PTU, we examined the immediate, direct product of $P450_{scc}$, pregnenolone. After inhibiting the function of 3β-HSD by trilostane, we examined the pregnenolone accumulation in rat Leydig cells. We found that 25-OH-C-stimulated (both 10^{-7} M and 10^{-5} M) pregnenolone production was inhibited by PTU, supporting a reduction in the function of $P450_{scc}$ (Fig. 4). It was interesting that pregnenolone production was reduced by about 40% by PTU when 25-

OH-C (10^{-5} M) was present, and by about a 90% reduction in testosterone production with 25-OH-C (10^{-5} M) as the substrate. Because 3β-HSD was inhibited by trilostane, we examined the pregnenolone accumulation rather than its production.

In order to investigate the mechanism by which PTU inhibits $P450_{scc}$ function, we challenged Leydig cells with serial doses of 25-OH-C. The kinetic analysis of 25-OH-C-treated Leydig cells revealed that $P450_{scc}$ has an apparent K_m of 2.96 μM and a V_{max} of 2.91 ng per h per 2×10^5 cells. The V_{max} of the PTU group was almost the same as the control group, but the K_m was 6.45 μM, which was almost 2.2-fold of the control value. This was consistent with a competitive inhibition mechanism (Fig. 5), and indicates that PTU might interfere with the formation of the binding complex of $P450_{scc}$ and cholesterol, or inhibit the function of $P450_{scc}$ in situ in Leydig cells.

In all species, the rate-limiting step in androgen biosynthesis is conversion of cholesterol to pregnenolone by $P450_{scc}$. In addition to this important enzyme, another protein, StAR, has been identified as being involved in the acute regulation of steroid production in steroidogenic tissues. The StAR protein represents a most attractive candidate for the transfer of cholesterol from cellular stores to the inner mitochondrial membrane. Based on data obtained from the regulated expression of StAR and the observed accompanying increase in steroid biosynthesis [36, 37], we propose that StAR is rapidly synthesized in the cytosol in response to hormone stimulation and is quickly targeted to the mitochondria via a specific receptor on the mitochondrial outer membrane. After transferring cholesterol into mitochondria, StAR would be catabolized. In the present studies, the protein and mRNA expressions of both $P450_{scc}$ and StAR were examined by Western blot and a semiquantitative RT-PCR assay. Based on our Western blot data, no significant differences were observed in StAR and $P450_{scc}$ protein expressions between the PTU and control groups after Leydig cells were incubated with PTU for 2 h (Fig. 6). Because our results indicated that PTU diminished testosterone production, this suggests that the acute inhibitory effects of PTU on testosterone production may be an inhibition of $P450_{scc}$ function, rather than an effect on $P450_{scc}$ protein expression.

Figure 7 shows that relative quantitation is indeed feasible for each individual expression because the housekeeping reference gene of choice, the ribosomal protein, L19 [38, 39], could be used as a reference gene for investigating StAR and $P450_{scc}$ expression [26]. Under the challenge of PTU, StAR mRNA expression was rapidly inhibited in 15 min, which would suggest that PTU may rapidly regulate StAR expression. Inasmuch as PTU inhibited StAR expression at the mRNA level but not at the protein level, we believe that PTU may prevent the translocation of StAR to mitochondria, which induces catabolism of StAR. However, no similar effect was detected for $P450_{scc}$ mRNA expression within 1 h (data not shown), suggesting that other mechanisms rather than $P450_{scc}$ mRNA expression may be involved in the PTU effect.

In summary, the present results demonstrate that PTU can act directly on rat Leydig cells to inhibit testosterone production. PTU rapidly diminished $P450_{scc}$ function and also the mRNA expression of StAR, and that this modulates the steroidogenesis of testosterone.

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